

IMMUNOHISTOCHEMICAL EXPRESSION OF EPIDERMAL GROWTH FACTOR RECEPTOR IN HEPATOCELLULAR CARCINOMA

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ABSTRACT

Introduction: Epidermal growth factor receptor (EGFR) signaling plays an important role in various cancers, including hepatocellular carcinoma (HCC). We aimed to evaluate immunoexpression of EGFR in HCC and surrounding non-tumor liver tissue and to correlate it to multiple clinicopathologic data.

Material and Methods: We analyzed 60 patients with HCC for multiple clinicopathologic characteristics and survival.

Presence of the immunosignal and the percentage of positive tumor cells at the whole tumor tissue sample and adjacent cirrhotic liver tissue were semi-quantitatively determined.

Results: Nineteen patients (31.67%) were female and 41 (68.33%) were male ranging in age from 31 to 85 years, median 61.88±10.51.

Mean survival time for female patients was 8.86±1.76 months, for male 13.03±1.50 months and overall survival was 11.6051±1.19 months.

The most patients had: T2 status (41.67%), no enlarged lymph nodes (90%), vascular invasion (63.33%) and well differentiated (43.33%) tumors.

EGFR immunoexpression was determined in range from 0% to 100% in both tumor and non-tumor tissue with mean value of 39.58% in tumor and 86.86% in cirrhotic tissue (p<0.00).

Higher percent of tumor EGFR positive cells were found in cases with higher T status, higher levels of AFP and poorly differentiated carcinoma, but not significantly.

Lower percent of tumor EGFR positive cells were found in patients with vascular invasion and enlarged lymph nodes, but also not significantly.

EGFR expression in tumor tissue significantly influenced survival of the patients (p<0.05).

Conclusion: The study showed that expression of EGFR in lower percentage of tumor cells was associated to favorable prognosis, making it a potential prognostic marker and therapeutic target.

Keywords: EGFR, HCC, cirrhosis, survival, immunohistochemistry

INTRODUCTION

Hepatocellular carcinoma (HCC) representing the fifth most common cancer and the third most common cause of cancer related mortality is a cancer with rising incidence in many countries in the past few decades [1,2]. HCC is a cancer that shows different incidence in various regions, between males and females, ethnic groups and races. It is a cancer strongly associated with few well known risk factors as hepatitis B and hepatitis C infection, exposition to Aflatoxin B1, alcoholic liver disease, non-alcoholic liver disease and other less common diseases as hemochromatosis, alpha1-antitrypsin deficiency, autoimmune hepatitis, some porphyries, diabetes and Wilson's disease [2-4]. The occurrence of HCC in most patients is associated with underlining liver cirrhosis, which is a result of long lasting chronic inflammation and fibrosis [5].

The liver response to tissue loss due to necrosis is coordinated by cytokines, growth factors and metabolites [6, 7]. The epidermal growth factor receptor (EGFR) signalling system is considered to play an important role in the regenerative and reparative liver response to the injury [8].

When liver injury and inflammation become chronic the regenerative mechanisms become dysregulated and some genetic alterations can occur, influencing cell proliferation. Cell proliferation can allow fixation of genetic mutation leading to development of preneoplastic and neoplastic nodules [9-12].

It is considered that EGFR is also involved in the pathogenesis and progression of different carcinomas including HCC [13-16]. There are reports in which EGFR expression in HCC is correlated to high proliferating activity, poor carcinoma differentiation, intrahepatic metastasis and bad prognosis [17, 18].

Prognosis of HCC depends on many factors such as the stage of the disease, morphological features of the tumor, nodal involvement, distant metastasis, disease recurrence, ability for surgical resection/transplantation, non surgical therapy and many others and despite different modalities of therapy it still remains poor [19].

EGFR targeted therapies, monoclonal antibodies and small molecule tyrosine kinase inhibitors have given some benefits in several types of tumors, including HCC, but new data and re-

search on EGFR are still needed to clarify its role in HCC carcinogenesis and to offer a base for new therapeutic strategies and possibilities [19, 20].

Detection of immunoexpression of EGFR in HCC has not attracted the attention of researchers in Republic of Macedonia recently.

The aim of this study is to evaluate immunoexpression of EGFR in HCC and surrounding non-tumor liver tissue and to correlate it to multiple clinical data and survival of the patients and pathologic characteristics of the tumor in order to evaluate the possible prognostic value of EGFR immunoexpression in patients from Republic of Macedonia.

MATERIAL AND METHODS

We analyzed 60 patients with histologically proven HCC, diagnosed and treated at the University Clinic of Gastroenterohepatology and the University Clinic of Abdominal Surgery in Skopje, Republic of Macedonia in a period of 6 years (2010-2016). There were 28 retrospective and 42 ongoing patients in the analyzed group of patients.

For the retrospective group we used the patient's medical files to find clinical data including demographic data, ultrasound and/or computed tomography images and laboratory status, from which we looked for the Alfa fetoprotein serum levels (AFP).

For the ongoing patients we made the same examinations as for the retrospective group of the patients.

Following parameters were determined by ultrasound and/or computed tomography images: the dimension of tumor node, the presence of tumor emboli in the large vessels, the presence of enlarged lymph nodes and the presence of cirrhosis in the surrounding liver tissue.

Any lymph node with a diameter of more than 1 cm was considered to be enlarged.

Serological tests for hepatitis B and C infection were performed in all patients. The stage of the disease was determined according to TNM classification (AJCC Cancer Staging Manual 2016). All patients were followed up from the date of HCC diagnosis till 24 months.

During the diagnostic procedure of HCC at the Institute of Pathology in Skopje, a grade

of differentiation, micro vascular invasion, T category of pTNM classification and the presence of cirrhosis in the surrounding, peritumoral liver tissue were determined.

Additional immunohistochemical stainings with an antibody against EGFR (Epidermal Growth Factor Receptor, Monoclonal Mouse, Anti-Human, Clone EGFR.25, Leica Biosystems, Novocastra™ Liquid, dilution 1:50) using Avidin-Biotin immunoperoxidase technique were made in all tumors (60 cases) and adjacent non-neoplastic liver tissue observed in 35 cases. For the visualization of the antigen-antibody reaction, LSAB and En-Vision kit from DAKO was used.

Presence and distribution of the signal, membranous and cytoplasmic and the percentage of positive tumor cells were evaluated. The percentage of the stained cells (regardless of the signal intensity) was semi-quantitatively determined in the whole tumor tissue sample at the slide and the whole non-tumor tissue at the slide, on microscopic fields at x200 magnification.

Two pathologists evaluated the slides separately in order to obtain objective findings of the immunoexpression of the EGFR.

Correlations of the EGFR immunoexpression and above listed clinical and pathological parameters were made.

For statistical analysis we used statistical software package Statistica 7.1 for Windows and SPSS Statistics 23.0, applying: Descriptive Statistics, Mann-Whitney U Test and Multiple linear regression. Statistical significance was accepted when P -values <0.05.

RESULTS

Nineteen patients (31.67%) out of 60 were female and 41 (68.33%) were male ranging in age from 31 to 85 years, median 61.88±10.51.

Mean survival time for female patients was 8.86±1.76 months, for male 13.03±1.50 and overall survival was 11.6051±1.19 months.

Clinicopathological features of the analyzed group are shown in Table 1.

Table 1. Clinicopathological features of patients and their tumors

Parameter	No of patients	%
Hepatitis		
No hepatitis	18	30
B	35	60
C	6	10
Cirrhosis		
No	8	13.33
Yes	52	86,67
Enlarged lymph nodes		
No	54	90
Yes	6	10
Vascular invasion		
No	22	36.67
Yes	38	63.33
Grade		
G1	26	43.33
G2	27	45
G3	7	11,67
T status		
T1	10	16.66
T2	25	41.66
T3	20	33.33
T4	5	8.33
AFP	min	max
	2,10	10000

EGFR immunoexpression was found in 53/60 (88.33%) of the tumor tissue samples and in 33/35 (94.28%) of non-tumor tissue samples and was determined in range from 0% to 100% in both tumor and non-tumor tissue with mean value of 39.58% in tumor and 86.86% in non-tumor liver tissue (p<0.00) (Table 2).

Table 2. Difference between EGFR expression in tumor and non-tumor tissue

Parameter	Rank Sum Tumor tissue	Rank Sum Cirrhotic tissue	U	Z	p-level	Valid N Tumor tissue	Valid N Cirrhotic tissue
EGFR %	2055.50	2504.50	225.50	-6.36	0.000	60	35

All samples of the non-tumor liver tissue were cirrhotic. Except the 2 EGFR negative cases, the percentage of EGFR positive cells in the cirrhotic tissue was in range from 70% to 100%

In the tumor tissue the percentage of EGFR positive cells was in range from 2% to 100% with more of the cases 31/53 (58.49%) with EGFR positive cells $\leq 50\%$, and 22/53 (41.50%) cases with EGFR positive cells from 51% to 100% (Figure 1).

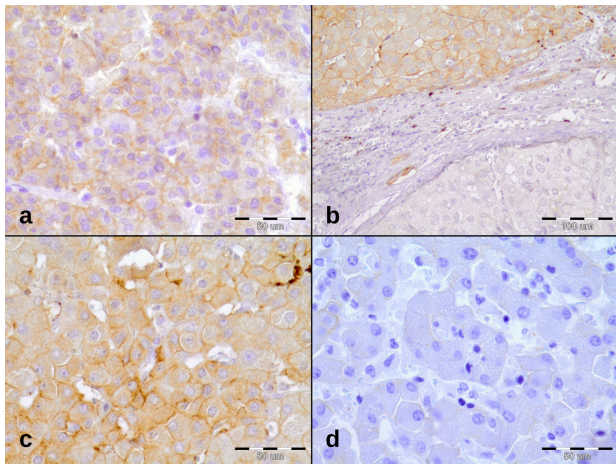


Figure 1. a) EGFR membranous and cytoplasmic immunorexpression in about 30% of tumor tissue (10x40) b) Approximately 100% membranous and cytoplasmic EGFR expression in cirrhotic liver tissue (upper half of the microphotography) and 0% expression in tumor tissue (lower half of the microphotography) (10x20) c) About 90% EGFR immunorexpression in the hepatocyte of cirrhotic liver (10x40) d) 0% EGFR immunorexpression in cirrhotic liver (10x40)

Expression of EGFR in tumor tissue was membranous in 30/53 (56.60%), cytoplasmic in 3/53 (5.66%) and combined membranous and cytoplasmic in 20/53 (37.73%) cases. The intensity of the signal was heterogeneous, from weak to strong, in different cases and in different areas in the same case.

Higher percent of tumor EGFR positive cells (regardless of the signal intensity) were found in patients with Hepatitis B infection, higher T status, higher levels of AFP and poorly differentiated carcinoma, but not significantly.

Lower percent of tumor EGFR positive cells were found in patients with vascular invasion and enlarged lymph nodes, compared to those who did not have vascular invasion and enlarged lymph nodes, but also not significantly.

Multiple linear regression analysis with EGFR as a dependant variable showed that the greatest influence to the EGFR expression, in descending order had: vascular invasion (Beta= -0.35) enlarged lymph nodes (Beta= -0.11), hepatitis B infection (Beta = -0.09), T3 status (Beta= -0.07) and the serum level of AFP (Beta= -0.02).

EGFR expression significantly influenced survival of the patients, and multiple linear regression showed that with each increase in patient's survival by 1 month the EGFR expression decreases by 1.63% ((B=-1.63) / (95%CI:-2.94 to -0.32) / $p < 0.05$).

DISCUSSION

Molecular mechanisms of oncogenic processes have been in the centre of attention of many researchers in the past few decades. Many cell signalling pathways are identified having an important role in tumor pathogenesis [21].

Wnt/ β -catenin, VEGF (Vascular endothelial growth factor), FGF (Fibroblast Growth Factor), MAPK (Mitogen-activated protein kinase), PI3k/AKT/mTOR (Phosphoinositide 3 kinase/mammalian target of rapamycin), EGFR (epidermal growth factor receptor), TGF- β (transforming growth factor- β) pathway and other pathways have been indicated in the development of many tumors. Understanding molecular mechanisms in carcinogenesis allows development of personalized target therapies that act directly and specifically on the components which regulate tumorigenesis [21].

The epidermal growth factor receptor also known as ErbB1 or HER-1 is a transmembrane protein receptor, which is a member of the ErbB family tyrosine kinase receptors (TKRs) [22]. It consists of an extracellular, a transmembrane and an intracellular domain, with tyrosine kinase domain and carboxy-terminal tail. Ligands that bind to extracellular domain of EGFR include epidermal growth factor (EGF), transforming growth factor α (TGF- α), amphiregulin (AR), epiregulin (EREG), betacellulin (BTC), heparin-binding EGF (HB-EGF) and epigen (EPGN) [23-26].

Upon ligand binding, EGFR can form homo- or heterodimers with other EGFR family members. Ligand binding activates the intrinsic kinase domain which initiates complex downstream signaling cascades, leading to the activation of diverse signaling pathways, controlling predominantly proliferation, differentiation, and survival [26].

It is considered that EGFR signaling pathway plays a key role in the liver response to the injury and that it takes a part in substantial and extensive crosstalk with other signaling pathways, modulating inflammation and cell prolifer-

eration. EGFR signaling pathway acts as a signaling centre for other growth factors, cytokines and inflammatory mediators [8].

Hepatocytes show high levels of EGFR [27] and overexpression of various EGFR ligands in liver with chronic injury is reported in experimental animal's models and humans [28]. It is also reported that EGF expression increases during cirrhosis and EGFR is overexpressed in human cirrhotic liver [29, 16], as we have found in our study.

Epidemiological studies and genetic experimental models findings of cancer showed that there was an association between chronic inflammation and cancer, and that inflammatory conditions were present before a neoplastic change occurred in some cancers [31]. HCC is an example of an inflammation related cancer. Liver inflammation and cirrhosis associated with liver regeneration after tissue damage caused by hepatitis infection, toxins or metabolic influences, are one of the main pathologic mechanisms of HCC carcinogenesis [5, 8, 10, 12, 28]. Beside the role EGFR pathway has in the liver response to the injury its altered activity is involved in the development and growth of HCC [8, 9, 12, 32].

Overexpression of EGFR occurs in about 40%-70% of conventional HCCs and is associated with more aggressive liver tumors, metastasis and poor patient survival [17, 18, 21, 26, 32-34]. In our study we found that overexpression of EGFR significantly influenced survival of the patients ($p < 0.05$), a finding that is consistent with other authors' reports.

We have also detected various expressions of EGFR in both cirrhotic liver tissue and HCC tissue in high percentage of cases: 88.33% and 94.28% respectively. Two cases of cirrhotic liver tissue and 7 cases of HCC tissue were completely negative for EGFR immunorexpression. The immunorexpression of EGFR in tumor and non-tumor tissue was significantly different with median higher overexpression in cirrhotic liver compared to HCC tissue. The range of EGFR immunorexpression in tumor tissue varied widely from case to case (2% to 100% positive cells) and was not significantly correlated to clinicopathological parameters. However, we found higher percent of tumor EGFR positive cells associated with higher T status, higher levels of AFP and poorly differentiated carcinoma and lower percent of tumor EGFR positive cells in patients with vascular invasion and enlarged

lymph nodes, but not significant. Our findings may correspond to the findings of DeCicco et al., who reported that carcinogen-mediated changes in EGF binding levels are different during the multistage process of hepatocarcinogenesis, and while early lesions of HCC show EGFR overexpression, advanced and differentiated HCCs tend to lose their EGFR overexpression [35].

Similar findings of the EGFR immunorexpression in HCC reported Panvichian R et al. who have found EGFR immunorexpression in only 32.5% of the examined cases, but did not find significant association between EGFR mutations and EGFR overexpression in HCC tissue, nor tumor differentiation [36].

It is also reported that the overexpression of EGFR present in the majority of HCCs does not correlate with an increase in EGFR gene copy number [33].

Beside the reports in which a correlation between EGFR expression and clinicopathologic characteristics of HCC were found there are studies, like ours, in which the findings did not show significant correlations [32, 33, 35].

These variations in EGFR expression and different findings in the EGFR correlation and clinicopathological characteristics suggest that EGFR role in carcinogenesis including HCC carcinogenesis is still not completed and its exact role is unknown.

Recently Ali R and Wendt MK promoted an "EGFR paradox" hypothesis in which they propose a change (switch) of EGFR function in breast primary tumor and metastatic cancer, with aberrant EGFR signalling initiating oncogenic transformation and primary tumor growth but inhibiting metastatic progression in association with downregulation of EGFR and shunting of EGFR away from the cell surface [37]. The study indicates that more research on EGFR is still needed in various types of cancer.

The EGFR overexpression that was detected in many epithelial cancers including HCC [39] was the reason for developing molecular target therapies, acting on the components of signaling pathways [40]. These therapies showed clinical benefits in some types of cancers, such as breast, colorectal and lung cancers [21].

Inhibiting EGFR with small molecular EGFR tyrosine kinase inhibitors and anti-EGFR antibodies has shown results in cell line and animal studies [26]. Some of them have been ap-

proved for human use, but the obtained results did not approve the expectations and only modest effects were achieved [26, 38, 39].

So, HCC remains a complex and heterogeneous tumor with a poor 5 year survival rate. Many patients with advanced disease in which surgical resection cannot be performed because of tumor size, location, or because of the patient's poor liver function have limited standard of care [40].

In our study we found that immunoexpression of EGFR significantly influenced the patient's survival. Additional studies, from different regions and different laboratories are important for analyzing EGFR expression in patients with HCC for giving opportunities for developing better strategies for molecular target therapies.

Further understanding of liver carcinogenesis and molecular alterations may be a base for developing more effective anticancer therapy for the patients with HCC.

Compliance with Ethical Standards

Authors declare that they have no conflict of interest.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Резиме

ИМУНОХИСТОХЕМИСКА ЕКСПРЕСИЈА НА РЕЦЕПТОР НА ЕПИДЕРМАЛЕН ФАКТОР НА РАСТ КАЈ ХЕПАТОЦЕЛУЛАРЕН КАРЦИНОМ

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Вовед: Сигналниот пат на рецепторот на епидермалниот фактор на раст (EGFR) игра важна улога кај различни типови рак, вклучувајќи го и хепатоцелуларниот карцином (HCC). Целта на овој труд е да се процени имуноекспресијата на EGFR кај HCC и на околното нетуморско ткиво и таа да се корелира со повеќе клиничко-патолошки карактеристики и со преживувањето.

Материјал и методи: Анализираме 60 пациенти со HCC за повеќе клиничко-патолошки карактеристики и во однос на преживувањето на пациентите. Дополнително, семиквантитативно го одредивме присуството и процентот на позитивните клетки во туморското и во околното циротично ткиво на целиот хистолошки пресек.

Резултати: Деветнаесет пациенти (31,67 %) беа од женски и 41 (68,33 %) пациент од машки пол, на возраст помеѓу 31 и 85 години, со средна возраст $61,88 \pm 10,51$.

Средното време на преживување кај жените беше $8,86 \pm 1,76$ месеци, кај мажите $13,03 \pm 1,50$, а вкупното – $11,6051 \pm 1,19$ месеци.

Повеќето пациенти имаа T2-статус на локален туморски раст (41,67 %), имаа васкуларна инвазија (63,33 %), добро диференцирани тумори (43,33 %) и немаа зголемени лимфни јазли (90 %).

Имуноекспресијата на EGFR беше одредена во опсег од 0% до 100 % и во туморското и во околното циротично ткиво со средна вредност од 39,58 % во туморското и 86,86 % во циротичното ткиво ($p < 0,00$).

Повисок процент на EGFR позитивни клетки беше најден кај случаите на HCC со повисок T-статус, повисоко ниво на алфа-фетопротеин и полошо диференциран карцином, но не сигнификантно. Понизок процент на позитивни клетки беше најден кај случаите со васкуларна инвазија и зголемени лимфни јазли, но исто така, несигнификантно. EGFR-експресијата во туморското ткиво сигнификантно влијаеше на преживувањето на пациентите ($p < 0,05$).

Заклучок: Оваа студија покажа дека имуноекспресијата на EGFR во понизок процент кај туморските клетки е здружена со подобра прогноза, што го прави EGFR потенцијален прогностички маркер и терапевтска таргет-молекула.

Клучни зборови: EGFR, HCC, цироза, преживување, имунохистохемија